

Peer Review Information

Journal: Nature Immunology

Manuscript Title: Transcriptome Dynamics of CD4⁺ T cells during Malaria Maps Gradual Transit from Effector to Memory

Corresponding author name(s): Ashraful Haque, Sarah A. Teichmann

Reviewer Comments & Decisions:

Decision Letter, initial version:
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Subject: Decision on Nature Immunology submission NI-RS29275

Message: 18th Mar 2020

Dear Dr. Haque,

Your Resource, "Transcriptome Dynamics of CD4⁺ T cells during Malaria Maps Progressive Transition from Effector to Memory." has now been seen by 2 referees. You will see from their comments copied below that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be very interested in considering a revised version that addresses these serious concerns.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions. Please do not hesitate to get in touch if you would like to discuss these issues further.

If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

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* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Resource format instructions at <http://www.nature.com/ni/authors/index.html>. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit <http://www.springernature.com/orcid>.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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Referee expertise:

Referee #1: Systems immunology, malaria

Referee #2: T cells, malaria

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

This excellent work ascertains the developmental path of T cell subsets in an animal model of Plasmodium infection, focusing more specifically on the fate of memory phenotypes. A transgenic CD4 T-cell clone that spontaneously differentiates in Th1 and Tfh effectors was adoptively transferred in the animal prior to infection. The authors also elegantly employed intermittent treatment with antimalarials to boost the development of memory cells that mimics a phenomenon that has been observed in humans. Droplet-based scRNAseq was used to map developmental trajectories, with a web application providing readers with the opportunity to look up genes of interest interactively. ATACseq was employed to measure chromatin accessibility in precursor, memory and effector cells.

This is a very interesting study overall and an impressive amount of work (and data!). However, it should be noted that it follows and builds upon an earlier work published a couple of years ago employing the same experimental model (including the same transgenic cells) and similar approaches (scRNAseq) [1]. The work at the time focused on characterizing the bifurcation between Th1 and Tfh effectors and did not investigate the development of memory phenotypes. The number of cells profiled was much lower since it relied on less advanced technology (Fluidigm C1 rather than droplet-based scRNAseq). ATACseq was also not used at the time. In any case, building and expanding upon prior work is generally a sound practice. It just seems that for context and completeness this prior work should figure much more prominently in the introduction and discussion. This would provide readers with a better sense of the extent of the knowledge gap being addressed by the study – which is also generally a good practice.

Along the same lines, it would also be appropriate to cite and discuss the work of Waickman et al. [2]. This group employed scRNAseq and longitudinal TCR clonotype analysis to investigate the commitment of T cells to the memory repertoire in the context of dengue virus vaccination. They also functionally characterized an effector/memory-associated transcriptional signature, which they found to be dominated by a metabolic transcriptional program.

Indeed, the authors of the present work could have deployed more efforts towards the functional interpretation of signatures and identification of pathways implicated in the development of memory phenotypes (and possibly confirming or extending the findings of Waickman and colleagues. The web application providing users access to profiling

information is in that sense useful but largely leaves readers to their own device when it comes to figuring out the functional significance of the changes being measured. Also, more efforts could be made in that sense.

It would also be interesting to have more of a discussion about the relevance and translation of the findings being reported in humans (some indication are given but more could be done in this sense). As elegant as the work in animal models is it would be important to keep this in perspective. Specifically, what would the implications be in terms of the design of vaccine strategies?

Finally, the authors seem intent to deposit data in a public repository, which definitely contributes to raising the impact of this contribution. The deposition should be verified, and accession IDs provided before final acceptance.

[1] 1: Lönnberg T, Svensson V, James KR, Fernandez-Ruiz D, Sebina I, Montandon R, Soon MS, Fogg LG, Nair AS, Liligeto U, Stubbington MJ, Ly LH, Bagger FO, Zwiessele M, Lawrence ND, Souza-Fonseca-Guimaraes F, Bunn PT, Engwerda CR, Heath WR, Billker O, Stegle O, Haque A, Teichmann SA. Single-cell RNA-seq and computational analysis using temporal mixture modelling resolves Th1/Tfh fate bifurcation in malaria. *Sci Immunol.* 2017 Mar 3;2(9). pii: eaal2192. doi: 10.1126/sciimmunol.aal2192. Erratum in: *Sci Immunol.* 2018 Mar 9;3(21):. PubMed PMID: 28345074; PubMed Central PMCID: PMC5365145.

[2] Waickman AT, Victor K, Li T, Hatch K, Rutvisuttinunt W, Medin C, Gabriel B, Jarman RG, Friberg H, Currier JR. Dissecting the heterogeneity of DENV vaccine-elicited cellular immunity using single-cell RNA sequencing and metabolic profiling. *Nat Commun.* 2019 Aug 14;10(1):3666. doi: 10.1038/s41467-019-11634-7. PubMed PMID: 31413301; PubMed Central PMCID: PMC6694189.

Reviewer #2:

Remarks to the Author:

In this manuscript, Soon et al. set out to understand the molecular dynamics underlying the development of memory CD4 T cells in response to experimental malaria infection in mice, and how this memory development is influenced by administration of the anti-malarial drug sodium artesunate.

They make use of malaria-specific TCRtg CD4 T cells to evaluate the development of memory states irrespective of influences by differences in TCR avidity. Memory states are evaluated by scRNAseq, scATAC seq and functional assays. Differentiation trajectories of CD4 T cells are computationally modeled based on previously published data from the same group (until day 7 after infection), combined with new data (day 7-28 after infection). Transcriptional and epigenetic states are furthermore explored in additional datasets obtained from a single memory phase timepoint. The authors provide a GUI to visually explore expression dynamics in their differentiation trajectory model, which is, like the scRNAseq and ATAC dataset themselves, a very nice resource for the community.

The value of this study lies primarily in the nice datasets acquired with a variety of different methods (plate-based scRNAseq, droplet-based scRNAseq, scATACseq, and clonal information based on endogenous TCRA chain sequences), and in the comparison of CD4

memory development with and without anti-malaria treatment. The finding that anti-malaria treatment increases the functionality (increased % IFN γ producers & reduced expression of exhaustion-associated genes) of the albeit reduced numbers of CD4 memory cells, and that it reduces their average state of exhaustion is interesting and novel.

The datasets generate many hypotheses, some of them consistent with previous studies, others new, but only few of them are assessed experimentally.

Specific points:

Due to the large variability in T cell subset definitions across labs, and in line with what has been agreed upon during the T cell nomenclature workshop at the recent T cell memory Keystone conference in Banff, please provide a section in the materials and methods that states how you throughout your manuscript define the various T cell subsets that you mention. This should include 'effector', 'memory', 'Tcm', 'Tem', 'Tfh', 'Th1', 'Tr1' etc. As an example, the sentence starting on row 310 is difficult to interpret since it is not very clear how the authors define 'effector' and 'memory' here.

The authors conclude that there are no detectable CD4 Tcm precursors at the peak of experimental malaria infection, and that therefore Tcm precursors are not essential for CD4 memory development. I do not think the presented data fully support this conclusion. In fact I interpret their data with regards to CCR7/CD62L quite differently, which leads me to a hypothesis that I find much more exciting than what the authors conclude. The authors show that none of their clusters (figure 2) is enriched for genes from a published expression signature (Ciucci et al). However, there are plenty of cells within each of the clusters that express genes from this signature. To me this shows that Tcm precursors may be formed in all of these clusters, i.e. irrespective of whether the CD4 is biased towards Th1 or Tfh. Originally, Tcm were defined in humans based on the expression of CCR7 that allows the cells to enter LNs through HEV (i.e. a migratory property). It seems quite plausible to me that Th1 and Tfh cells can have the possibility to either exist in a CCR7+ state that allows LN entry through HEV, and in a CCR7- state that does not allow that, and the presented expression data are in line with that. In fact, I would find this a quite interesting finding. Furthermore, such an observation that LN-homing cells exist among both Th1 and Tfh is not in contradiction with their data that support a linear relationship of naïve to Th1/Tfh effector to memory. Some of the Th1 and some of the Tfh effectors may have LN-homing properties through HEV, and then may continue to live as memory cells that still retain their Th1/Tf1 bias. By no means does HEV/LN homing potential (often designated as Tcm) need to be a mutually exclusive property to any of the other CD4 properties that have gotten subset names like Th1 and Tfh. The notion that a cell can only belong to one 'subset' is in my opinion a too dogmatic view on T cell subsets, where it is often forgotten how these subset names originally came to be. The Tcm denomination originally just referred to the property to home to LN through HEV and can therefore absolutely co-exist with the property of a cell to help B cells (Tfh) or not, or to be good IFN γ producers (Th1).

Since it is possible that the published Tcm precursor signature contains genes that are biased towards a viral infection, it would be informative to see how the expression patterns of Ccr7 and Sell (both are required for HEV entry) relate to the identified clusters. Are these individual genes also uniformly divided over all clusters, or somewhat more skewed to the one or the other? These data are only provided much later in S7A, and 'buried away' in the supplemental materials with barely a description of expression

patterns and a weak conclusion 'while ... Tfh and Ccr7+ Tcm might emerge within the same Tfh pathway, the GPfates model had insufficient power to differentiate between them'. I think their data are much more interesting than that! To me their data suggest that cells with capacity to home to LN through HEV are selectively present among cells with Tfh phenotype, and not among Th1. While Sell is detected in both Th1 and Tfh, Ccr7 is only detected in Tfh, and since co-expression of both molecules is required for HEV passage, only the Tfh cells contain at least a fraction that can also follow this migratory path. Given the high drop-out rate in scRNAseq data, the co-expression of Sell and Ccr7 on RNA level in ~10% of the cells is quite notable. I would encourage the authors to rethink their interpretation of the CCR7 data and further experimentally explore the hypothesis that only Tfh but not Th1 memory cells have the capacity to home through LN's via HEVs. This could for example be done by adoptive transfer short-term homing experiments in the presence or absence of MEL-14. If such experimental data would confirm that only Tfh but not Th1 memory cells have HEV/LN homing capacity, that would substantially add to the novelty and relevance of this study.

The authors present flow cytometry data with a CCR7 antibody. Since they only see detectable staining on naïve T cells, they conclude that the antigen-experienced cells at day 7 do not contain Tcm cells. Unfortunately, the currently commercially available antibodies to CCR7 are quite famous for being of so poor quality that they can only ever positively stain naïve T cells, which express much much higher levels of CCR7 than Tcm. Therefore, undetectable staining of antigen-experienced murine T cells cannot be considered evidence of absence of CCR7 expression, unless there is a positive control of antigen-experienced (not naïve) murine T cells that do show CCR7-staining.

Id2 is identified as a transcription factor associated with late pseudotime irrespective of Th1/Tfh state and therefore interpreted as being 'associated with memory onset'. How then do the authors interpret their flow cytometry data showing that Id2 levels are highest on day 7, the peak of the effector response, so much earlier than the onset of memory, and that during memory (d28), the expression levels are lower again (Fig 4E)? To me the flow data do not support the conclusion that Id2 expression is associated/correlated with memory onset. What might also help is a clear description of how the authors define 'memory onset'. Similarly, cMaf (Fig 4F) is expressed at higher levels at day 7 than 28 – to me this does not argue for a 'role for cMaf in CD4 memory in malaria'. In relation to this, in Figure 4E the gates are very different for day 7 than all other days, which means that comparing the resulting cell frequencies between day 7 and the other days is somewhat misleading. Given that these transcription factors do not identify clear + and – populations, but rather present a shift in mean expression levels, I would find it more appropriate to evaluate the shifts in Id2, TCF1 and cMaf expression (4E+F) by (geo)MFI, rather than by % positives. The same applies to Fig 5G.

S2E+F: How do the authors interpret their finding that activation of naïve T cells leads to a lower % of KI67+ cells in the presence of IAT than saline. Does IAT reduce responses of naïve T cells? Is that a desirable effect of the drug?

The clonal analysis based on endogenous TCRab sequences is a beautiful system, but also comes with caveats. The authors mention that 'as expected, family members did not cross timepoints or conditions (data not shown)'. I believe this is a very crucial control if one wants to conclude on clonal overlap between fates. Therefore, I would pledge for the data of this control to be shown.

Since the chances that a single family has detectable members in both the Th1 and Tfh fate is higher when the number of detected families is higher, I would like to see whether the authors' conclusion with regards to Fig 5D holds true if only families are depicted that have an equal number of detected members. So, for example when only those families are plotted that have two detected members (and only those that have 3 members – if these are enough families to assess).

To address whether 'Th1 or Tfh effectors surviving contraction remained on their apparent Th1 or Tfh developmental trajectories with no lineage plasticity', the authors track the fate of adoptively transferred d7 sorted Tfh and Th1. The subsets are transferred into mice receiving IAT, and not into mice receiving saline, because, as the authors state, in the IAT group 'apparent developmental trajectories [inferred from scRNAseq data] were better preserved over time'. Exactly for that reason, I think it is crucial to transfer the subsets (additionally) into the saline group recipients, to avoid any potential selection bias. The cells transferred into the IAT treated mice partially retained their respective Th1/Tfh phenotype, but is this just because they were exposed to IAT? Or does this also happen in the 'natural' untreated setting? The scRNAseq data in the saline group show more convergence of Tfh/Th1 RNA profiles towards each other at later pseudotime. Is this indicative of fate plasticity in the absence of IAT? Does IAT limit plasticity? This can be addressed by adoptively transferring the cells into mice that will not be treated with IAT vs those that will be treated. I think such an experiment would strengthen the manuscript substantially.

How do the authors explain the loss of CXCR5 expression on most of the sorted CXCR5+ cells after transfer? Did most of the transferred Tfh convert into an undefinable state, while only a minority (those that retained CXCR5) remained faithful to their Tfh fate? In that regard, the IFN γ production is quite variable in the CXCR5+ transfer group. Can this be explained by analyzing a mixture of 'true' Tfh that kept their Tfh fate, mixed with some undefined other CD4 state? How is IFN γ production if the CXCR5+ transfer group is stratified into those cells that kept their CXCR5 expression and those that lost it?

In 6F, please show the gate that defines whether a cell is considered Tigit+ or –

6C: What is cluster 8? Please comment

6E/G: For completeness I would like to see the scores for all clusters, and the expression patterns (like in 6D) of the individual genes making up the scores, in particular if the scores are just made up of a few genes (like Tcm)

Row 419: 'IAT had significantly improved the quality of ...memory responses....by boosting Tcm and CG Tfh responses'. Why is it better for the quality of the response to boost Tcm / CG Tfh responses? In order to make the strong point that IAT improves the quality of the memory response, a functional measurement of quality would be warranted. Are mice better protected from a secondary malaria infection if they were treated with IAT vs saline after the primary infection?

Patterns of transcriptional and epigenetic changes from naïve to effector to memory states are quite well described for CD8 T cells (e.g by groups of Ben Youngblood, Ananda Goldrath, Rafi Ahmed). These studies also found that numerous epigenetic changes that take place after T cell priming are retained from effector to memory phase. I would find it appropriate to discuss these.

The authors suggest that anti-malarial drug treatment of humans in endemic regions might serve to support the generation of parasite-specific CD4 memory. Their experiments investigated the effect of anti-malarial drug treatment after primary infection. In endemic regions humans are usually infected for the first time at an early age and will be infected much more often during the course of their life. Therefore, for endemic regions it is much more relevant to know whether anti-malarial treatment affects memory generation after repeated malaria exposures, or only after the first exposure. This could be experimentally addressed by evaluating the memory T cells generated after repeated malaria exposures, with IAT administered after a secondary response.

When discussing their finding that single naïve CD4 can give rise to both Tfh and Th1 fates, but not necessarily always produce both fates, I would find it appropriate to discuss that an earlier study has come to the same conclusion in another infection model (Marc Jenkins lab, Tubo et al).

When discussing TCF-1 expression in the Tfh lineage, it would be relevant to discuss parallels to CD8 T cells, since their model of persistent malaria infection results in a certain degree of T cell exhaustion, and since TCF-1 plays a crucial role in CD8 differentiation in the context of persistent viral infections (e.g. LCMV clone 13) which also leads to exhaustion (e.g. groups of John Wherry, Dietmar Zehn, Rafi Ahmed)

Author Rebuttal to Initial comments
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We thank the Reviewers for comments and considered suggestions on our manuscript. While the COVID19 pandemic slowed our progress, we have addressed ALL concerns thoroughly, with new animal experimentation, computational analysis, and substantial additional comment in the Introduction and Discussion sections.

We present below a point-by-point response to the Reviewers' comments, with our comments in BLUE text. Wherever appropriate we present the new figures and new text that have been incorporated into the revised manuscript, as well as line numbers for ease of reference. Excerpts from the manuscript are presented as *italicized* and in "quotes".

Reviewer #1:

Remarks to the Author:

This excellent work ascertains the developmental path of T cell subsets in an animal model of Plasmodium infection, focusing more specifically on the fate of memory phenotypes. A transgenic CD4 T-cell clone that spontaneously differentiates in Th1 and Tfh effectors was adoptively transferred in the animal prior to infection. The authors also elegantly employed intermittent treatment with antimalarials to boost the development of memory cells that mimics a phenomenon that has been observed in humans. Droplet-based scRNAseq was used to map developmental trajectories, with a web application providing readers with the opportunity to look up genes of interest interactively. ATACseq was employed to measure chromatin accessibility in precursor, memory and effector cells.

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We have now inserted further comment in the Introduction (Lines 93-102), which places this new study in appropriate context with our previous study in *Science Immunology* 2017.

"From analysis of hundreds of single-cell transcriptomes, Th1 and Tfh effector clones were observed to emerge from a proliferating, glycolytic, intermediate state, with the balance of fates influenced extrinsically by monocytes or B cells. Due to assessment of a

relatively small number of cells, rarer sub-populations such as memory precursor cells were not detectable. In addition, we examined neither germinal centre (GC) responses, memory and exhaustion, nor the effect of anti-malarial drug treatment, an important tool for malaria control in endemic regions. Here, we have extended our previous work by studying thousands of parasite-specific CD4⁺ T cells over time in the spleen, with two complementary scRNA-seq platforms (higher-resolution and higher throughput), single-cell Assay for Transposase-Accessible Chromatin using sequencing (scATAC-seq), computational modelling and in vivo validation."

Along the same lines, it would also be appropriate to cite and discuss the work of Waickman et al. [2]. This group employed scRNAseq and longitudinal TCR clonotype analysis to investigate the commitment of T cells to the memory repertoire in the context of dengue virus vaccination. They also functionally characterized an effector/memory-associated transcriptional signature, which they found to be dominated by a metabolic transcriptional program.

Thank you for bringing Waickman *et al* to our attention. The study examines CD8⁺ T cell memory in humans vaccinated against dengue, with some parallels to be drawn with our work. Longitudinal TCR analysis showed that effector CD8⁺ T cells give rise to memory, with scRNA-seq suggesting the phenotype of memory precursors. Of two types of effector detected, only one appeared to give rise to memory. This appears to contrast with our model in which at least two types of memory cell emerge from two main effector types; yet this difference is likely due to the manner in which peptide-responding memory cells were isolated based on a CD69^{hi} CD25^(hi) phenotype only, when CD69^{hi} CD25^{lo} cells were left unexamined. In any case, we cite this reference in the Introduction as a longitudinal human study using scRNAseq to study memory in T cells:

(Lines 68-70) "In humans, longitudinal assessment of antigen-specific T cells, usually CD8⁺ T cells, for example after vaccination against mosquito-borne viral diseases, yellow fever and dengue, indicate that effector T cells give rise directly to circulating memory{Waickman, 2019 #803; Akondy, 2017 #526}"

Indeed, the authors of the present work could have deployed more efforts towards the functional interpretation of signatures and identification of pathways implicated in the development of memory phenotypes (and possibly confirming or extending the findings of Waickman and colleagues. The web application providing users access to profiling information is in that sense useful but largely leaves readers to their own device when it comes to figuring out the functional significance of the changes being measured. Also, more efforts could be made in that sense.

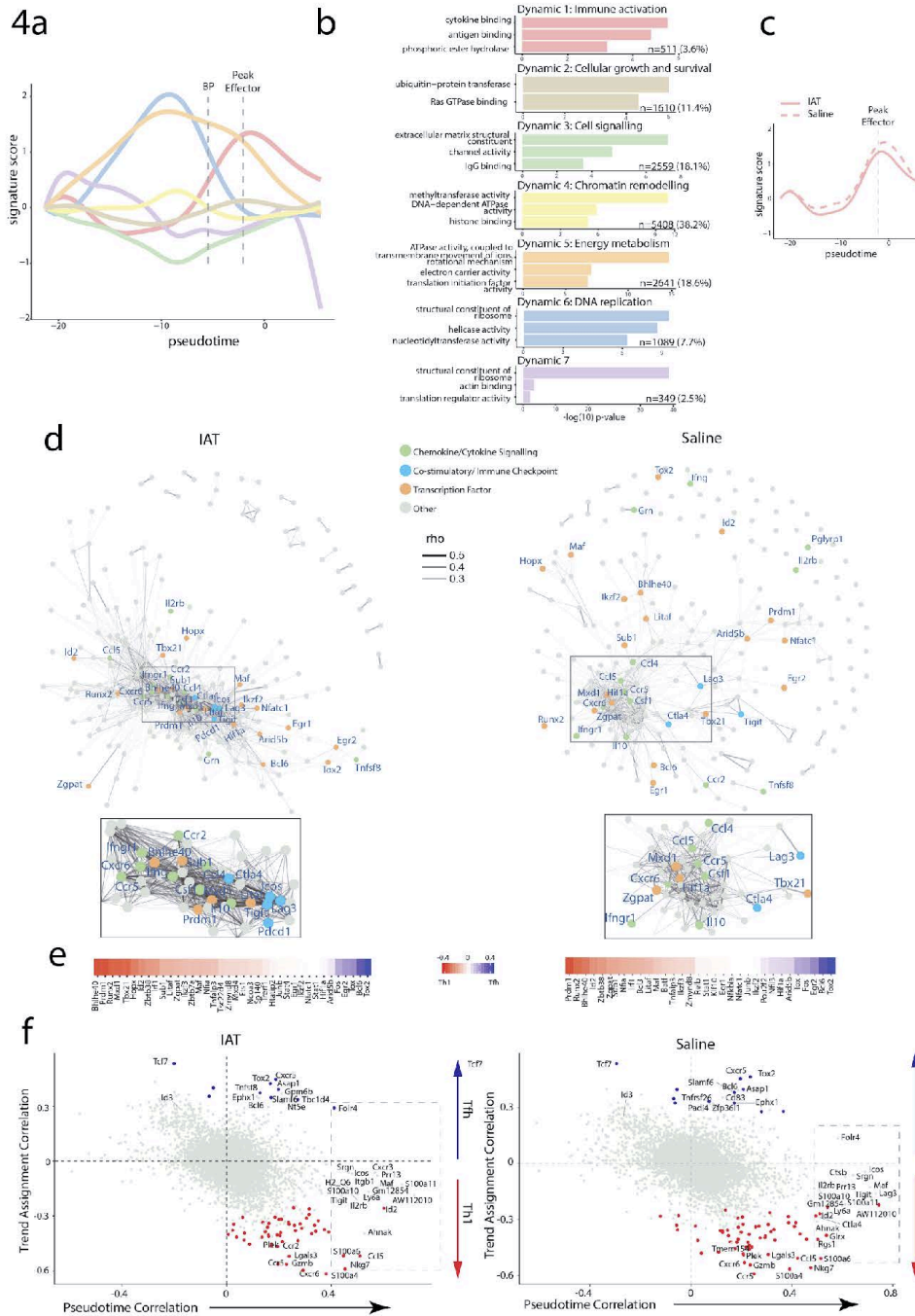
We have undertaken several new "functional interpretation" analyses at genome scale of the dynamics of gene expression accompanying effector to memory transition in CD4⁺ T cells, detailed below and culminating in a new Figure 4:

- 1) We used our recent computational approach, SpatialDE, published in *Nature Methods* (Svensson *et al* 2018) to identify groups of genes with similar patterns of

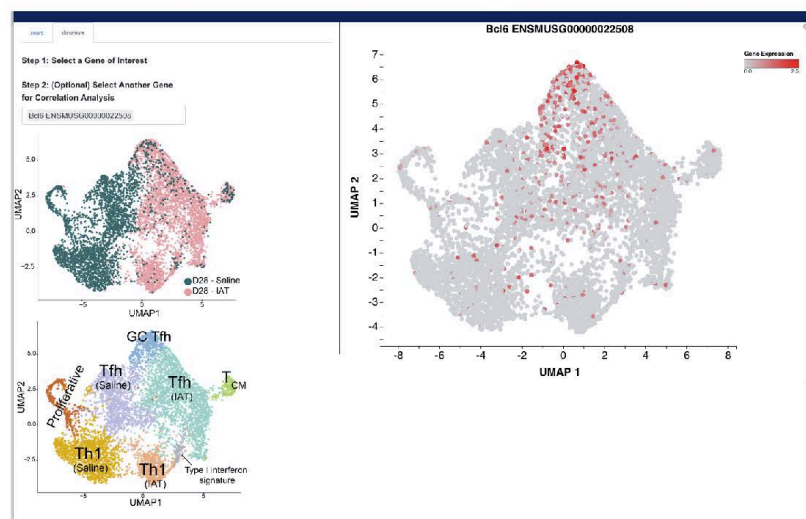
expression during progression from naivety to effector to memory. We categorised 14,167 genes (in IAT) & 15,310 genes (in persisting infection) in our GPfates models into seven different Dynamics. Some peak and drop away, while others drop away to recover towards the end of pseudotime. Notably, we define one Cluster of 3-4% of the genome whose expression peaks late, and is partially preserved in memory.

- 2) We employed Gene Ontology enrichment analysis to define the biological processes associated with these different clusters, which reveals how DNA replication, RNA processing, Protein translation, energy requirements, and immune-related gene expression all vary over time. We find that memory induction is overwhelmingly associated with immunological processes.
- 3) We employ Spearman correlation and network analysis to identify correlated gene families, and reveal that IAT treatment serves to enhance the Th1/Tfh axis amongst PbTII cells, whilst showing that immune checkpoint markers feature regardless of whether infection is treated or not.
- 4) Finally, our network analysis revealed expected and novel transcription factors associated with memory induction in CD4 T cells.

This body of analysis represents a substantial extension to the paper. Figure 4 has been re-vamped to incorporate this new work. We present below the new Figure 4, which contains: panels a and c) genome-wide Dynamics analysis, b) GO term enrichment analysis of Dynamics d) Spearman correlation and network analysis of genes preserved in memory, and e) a focus on transcription factors preserved in memory. This adds to the previous data in panel (f) showing correlation of memory-associated genes with either Th1 or Tfh trend assignments.



To facilitate better functional interrogation of our data by the research community, we have added a second feature to the Graphical User Interface (GUI): the capacity to interrogate the droplet-based “Memory” scRNA-seq dataset from Day 28. This allows researchers to view not only the dynamics of gene expression in the original visualisation, but also in an independent dataset more detail in gene expression in GC-Tfh cells, memory Tfh, Tcm, Th1-memory, and partially exhausted cells. We believe this brings experimental rigour to our GUI. We present below a screen shot of the new feature:



It would also be interesting to have more of a discussion about the relevance and translation of the findings being reported in humans (some indication are given but more could be done in this sense). As elegant as the work in animal models is it would be important to keep this in perspective. Specifically, what would the implications be in terms of the design of vaccine strategies?

We now include substantial paragraphs in the Discussion (Lines 543-550 & 556-566), which specifically deals with translational implications of our study:

“Currently, the only available malaria vaccine, RTS,S/AS01, generates relatively short-lived immunity against liver-stage parasites, which correlates with antibody production and CD4⁺ T cell production of IL-2 and TNF, but not IFN γ {White, 2013 #337; Olotu, 2011 #812; Ockenhouse, 2015 #813}. RTS,S/AS01 provides an example of malaria immunity dependent on antibodies, less so Th1 responses. Since Th1 cells alone can protect {Pinzon-Charry, 2010 #5030}, an optimal blood-stage vaccine strategy might be dual promotion of protective antibodies and Th1-memory. Our study highlights these two mechanisms can be elicited simultaneously amongst T cells of the same specificity during natural exposure, although development of one influences and may interfere with the other {Ryg-Cornejo, 2016 #6369; Obeng-Adjei, 2015 #6381}.”

“Given intermittent anti-malarial drug treatment protects individuals in malaria-endemic regions for at least one year after drug cessation {Muhindo, 2019 #7313;Schellenberg, 2005 #7317}, our data suggest reduced blood-stage parasite load has profound effects on the memory phenotypes and functional potential of parasite-specific CD4⁺ T cells. We speculate individuals in endemic regions could be treated with anti-malarial drugs to minimise deleterious effects of chronic infection on immune priming during natural exposure events or prior to receiving a malaria vaccine {von Seidlein, 2020 #814}. Another implication from our data for naturally-acquired or vaccine-mediated malaria immunity is that Th1-responses, which also promote Cxcr3, Ifng and Tbx21 along the Tfh lineage, should be avoided while antibodies are being generated, but could be promoted subsequently. To achieve this we propose separating the development of humoral and Th1-cellular immunity by a temporally-segregated priming regimen using distinct antigens.”

Finally, the authors seem intent to deposit data in a public repository, which definitely contributes to raising the impact of this contribution. The deposition should be verified, and accession IDs provided before final acceptance.

Data are in the progress of being deposited into ArrayExpress. We provide the accession number for the scRNA-seq data generated on the 10X Genomics platform (E-MTAB-9317). The remaining accession numbers will be provided upon acceptance of the manuscript.

[1] 1: Lönnberg T, Svensson V, James KR, Fernandez-Ruiz D, Sebina I, Montandon R, Soon MS, Fogg LG, Nair AS, Liligeto U, Stubbington MJ, Ly LH, Bagger FO, Zwiessele M, Lawrence ND, Souza-Fonseca-Guimaraes F, Bunn PT, Engwerda CR, Heath WR, Billker O, Stegle O, Haque A, Teichmann SA. Single-cell RNA-seq and computational analysis using temporal mixture modelling resolves Th1/Tfh fate bifurcation in malaria. *Sci Immunol*. 2017 Mar 3;2(9). pii: eaal2192. doi: 10.1126/sciimmunol.aal2192. Erratum in: *Sci Immunol*. 2018 Mar 9;3(21):. PubMed PMID: 28345074; PubMed Central PMCID: PMC5365145.

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In this manuscript, Soon et al. set out to understand the molecular dynamics underlying the development of memory CD4 T cells in response to experimental malaria infection in mice, and how this memory development is influenced by administration of the anti-malarial drug sodium artesunate.

They make use of malaria-specific TCRtg CD4 T cells to evaluate the development of memory states irrespective of influences by differences in TCR avidity. Memory states are evaluated by scRNAseq, scATAC seq and functional assays. Differentiation trajectories of CD4 T cells are computationally modeled based on previously published data from the same group (until day 7 after infection), combined with new data (day 7-28 after infection). Transcriptional and epigenetic states are furthermore explored in additional datasets obtained from a single memory phase timepoint. The authors provide a GUI to visually explore expression dynamics in their differentiation trajectory model, which is, like the scRNAseq and ATAC dataset themselves, a very nice resource for the community.

The value of this study lies primarily in the nice datasets acquired with a variety of different methods (plate-based scRNAseq, droplet-based scRNAseq, scATACseq, and clonal information based on endogenous TCRAb chain sequences), and in the comparison of CD4 memory development with and without anti-malaria treatment. The finding that anti-malaria treatment increases the functionality (increased % IFN γ producers & reduced expression of exhaustion-associated genes) of the albeit reduced numbers of CD4 memory cells, and that it reduces their average state of exhaustion is interesting and novel.

The datasets generate many hypotheses, some of them consistent with previous studies, others new, but only few of them are assessed experimentally.

Specific points:

Due to the large variability in T cell subset definitions across labs, and in line with what has been agreed upon during the T cell nomenclature workshop at the recent T cell memory Keystone conference in Banff, please provide a section in the materials and methods that states how you throughout your manuscript define the various T cell subsets that you mention. This should include 'effector', 'memory', 'Tcm', 'Tem', 'Tfh', 'Th1', 'Tr1' etc. As an example, the sentence starting on row 310 is difficult to interpret since it is not very clear how the authors define 'effector' and 'memory' here.

[This is now added in the Materials & Methods, also presented below for reference.](#)

Table 1. T cell terminology

	Definition
Naive CD4 ⁺ T cell	A mature CD4 ⁺ T cell yet to encounter cognate antigen presented in the context of MHCII, expressing CCR7 and CD62L
Effector CD4 ⁺ T cell	A CD4 ⁺ T cell exhibiting helper functions such as cytokine production, after being activated by cognate antigen, and having progressed through clonal expansion
Memory CD4 ⁺ T cell	A CD4 ⁺ T cell previously activated and clonally expanded via cognate antigen, which although quiescent, retains a capacity to respond to a second round of activation with cognate antigen.
Th1 cell	Effector CD4 ⁺ T cell expressing high levels of T-bet and IFN γ also marked by cell-surface expression of CXCR6
Tfh cell	Effector CD4 ⁺ T cell expressing high levels of Bcl6 and CXCR5, as well as ICOS, with a capacity to leave T cell zones and move to B cell follicles in secondary lymphoid tissue
GC Tfh cell	Effector CD4 ⁺ T cell expressing high levels of Bcl6, CXCR5 and PD1, with a capacity to interact with B cells in the germinal centre
Tr1 cell	Effector CD4 ⁺ T cell co-expressing IFN γ and IL-10
T _{CM} cell	Antigen-experienced, quiescent T cell that expresses CCR7 (with or without CD62L)
T _{EM} cell	Antigen-experienced, quiescent T cell which rapidly expresses cytokine and/or specific T helper phenotypes during recall

The authors conclude that there are no detectable CD4 Tcm precursors at the peak of experimental malaria infection, and that therefore Tcm precursors are not essential for CD4 memory development. I do not think the presented data fully support this conclusion. In fact I interpret their data with regards to CCR7/CD62L quite differently, which leads me to a hypothesis that I find much more exciting than what the authors conclude. The authors show that none of their clusters (figure 2) is enriched for genes from a published expression signature (Ciucci et al). However, there are plenty of cells within each of the clusters that express genes from this signature. To me this shows that Tcm precursors may be formed in all of these clusters, i.e. irrespective of whether the CD4 is biased towards Th1 or Tfh. Originally, Tcm were defined in humans based on the expression of CCR7 that allows the cells to enter LNs through HEV (i.e. a migratory property). It seems quite plausible to me that Th1 and Tfh cells can have the possibility to either exist in a CCR7+ state that allows LN entry through HEV, and in a CCR7- state that does not allow that, and the presented expression data are in line with that. In fact, I would find this a quite interesting finding. Furthermore, such an

observation that LN-homing cells exist among both Th1 and Tfh is not in contradiction with their data that support a linear relationship of naïve to Th1/Tfh effector to memory. Some of the Th1 and some of the Tfh effectors may have LN-homing properties through HEV, and then may continue to live as memory cells that still retain their Th1/Tfh bias. By no means does HEV/LN homing potential (often designated as Tcm) need to be a mutually exclusive property to any of the other CD4 properties that have gotten subset names like Th1 and Tfh. The notion that a cell can only belong to one 'subset' is in my opinion a too dogmatic view on T cell subsets, where it is often forgotten how these subset names originally came to be. The Tcm denomination originally just referred to the property to home to LN through HEV and can therefore absolutely co-exist with the property of a cell to help B cells (Tfh) or not, or to be good IFN γ producers (Th1).

Since it is possible that the published Tcm precursor signature contains genes that are biased towards a viral infection, it would be informative to see how the expression patterns of Ccr7 and Sell (both are required for HEV entry) relate to the identified clusters. Are these individual genes also uniformly divided over all clusters, or somewhat more skewed to the one or the other? These data are only provided much later in S7A, and 'buried away' in the supplemental materials with barely a description of expression patterns and a weak conclusion 'while ... Tfh and Ccr7+ Tcm might emerge within the same Tfh pathway, the GPfates model had insufficient power to differentiate between them'. I think their data are much more interesting than that! To me their data suggest that cells with capacity to home to LN through HEV are selectively present among cells with Tfh phenotype, and not among Th1. While Sell is detected in both Th1 and Tfh, Ccr7 is only detected in Tfh, and since co-expression of both molecules is required for HEV passage, only the Tfh cells contain at least a fraction that can also follow this migratory path. Given the high drop-out rate in scRNAseq data, the co-expression of Sell and Ccr7 on RNA level in ~10% of the cells is quite notable. I would encourage the authors to rethink their interpretation of the CCR7 data and further experimentally explore the hypothesis that only Tfh but not Th1 memory cells have the capacity to home through LN's via HEVs. This could for example be done by adoptive transfer short-term homing experiments in the presence or absence of MEL-14. If such experimental data would confirm that only Tfh but not Th1 memory cells have HEV/LN homing capacity, that would substantially add to the novelty and relevance of this study.

We acknowledge that the T_{CM} phenotype was originally coined based on the capacity to home to LN via CCR7 expression, and that this does not preclude cells from expressing either a Th1 or Tfh memory phenotype. Based on the gene signature alone, we understand the Reviewer's alternative interpretation, that perhaps all PbTII effectors have the capacity to give rise to Tcm cells, and have included mention of this in the manuscript (Lines 567-575) also shown here:

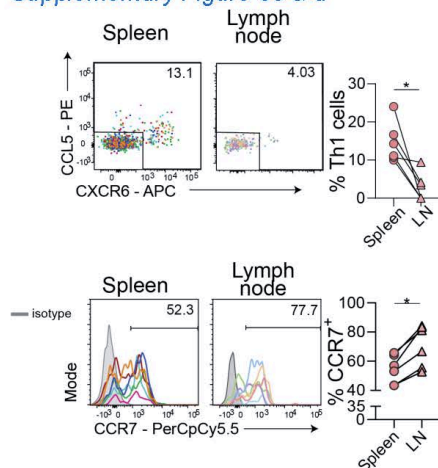
"In contrast to observations during LCMV infection{Ciucci, 2019 #7103}, we found no evidence of a distinct CCR7-expressing T_{CM} precursor in PbTII cells during the first week of Plasmodium infection, with a recent precursor gene signature being uniformly expressed amongst all effector PbTII. An alternative interpretation might be that all effectors expressed equal T_{CM} potential, although this appears unlikely given the highly

restricted nature of Ccr7 expression along one lineage in our dynamic modelling, and that effector fate-mapping suggested minimal cross-over between trajectories. Reasons for apparent discrepancies between LCMV and our model may be due to inherent differences in responses to viral versus parasitic infection{Arroyo, 2020 #610}, or in the specific characteristics of transgenic TCRs used."

When we examine the dynamics in our GPfates model, and the fate-mapping experiments conducted with CXCR5 or CXCR6 expressing effectors, our data support only one lineage, the CXCR5⁺ lineage, giving rise to T_{CM} cells, based particularly on the functional marker CCR7. To test the Reviewer's hypothesis that T_{fh}-lineage PbTII's but not Th1-lineage PbTII's can traffick to LN, we conducted further experimentation to compare both PbTII cell types at D28 p.i. for presence in inguinal lymph nodes versus the spleen. This new data is now presented in Supp Figure 8c and 8d and inserted in lines 384-387 of the manuscript, also reproduced below for reference. Our data is consistent with the hypothesis, showing that amongst PbTII cells, Th1 memory cells, though readily detectable in the spleen, were significantly less prevalent in inguinal LN. In contrast, CCR7⁺ cells were enriched among PbTII cells in LN compared to spleen. These new data suggest that CCR7⁺ non-Th1 memory cells exhibit the greater HEV/LN homing capacity:

Lines 384-387: *"We hypothesized therefore that T_{CM} cells in lymph nodes do not display a Th1 phenotype. Consistent with this, paired analysis across tissues revealed a significant drop in the proportions of memory PbTII's expressing a Th1 phenotype in inguinal lymph nodes compared to spleen, as well as a concomitant increase in those expressing CCR7 (Supplementary Figure 8c & d)."*

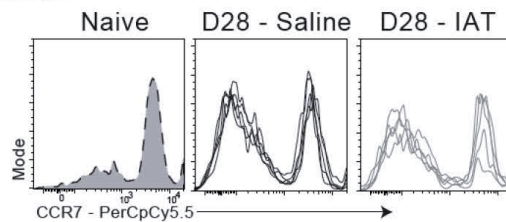
Supplementary Figure 8c & d



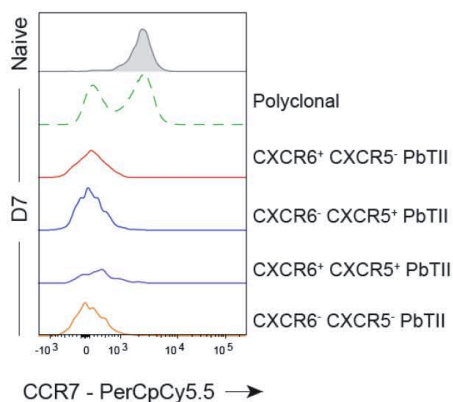
The authors present flow cytometry data with a CCR7 antibody. Since they only see detectable staining on naïve T cells, they conclude that the antigen-experienced cells at day 7 do not contain T_{CM} cells. Unfortunately, the currently commercially available

antibodies to CCR7 are quite famous for being of so poor quality that they can only ever positively stain naïve T cells, which express much much higher levels of CCR7 than Tcm. Therefore, undetectable staining of antigen-experienced murine T cells cannot be considered evidence of absence of CCR7 expression, unless there is a positive control of antigen-experienced (not naïve) murine T cells that do show CCR7-staining.

We bring to the Reviewer's attention CCR7 staining on PbTII cells at D28 p.i. As shown in Figure 1d (also shown below for reference) CCR7 expression is clearly present on a sub-fraction of antigen-experienced PbTII cells at this timepoint, illustrating that our stain works well. This is in clear contrast to the absence of expression seen on PbTII cells at D7 p.i.



To further illustrate the fidelity of our CCR7 stain, we present below for the Reviewer's benefit (ie. not in manuscript), a comparison of CCR7 expression on polyclonal CD4⁺ T cells versus PbTIIs in the same samples at day 7 p.i.. This reveals the extent of +ve and -ve CCR7 staining in these samples, and suggests that PbTIIs express little to no CCR7. Therefore, we feel our CCR7 stain is robust, and we maintain that a CCR7⁺ Tcmp population is absent in this experimental malaria model. Given the positive CCR7 staining of antigen-experienced PbTIIs provided in Figure 1d, we propose not to add further quality control data for CCR7-staining.



Id2 is identified as a transcription factor associated with late pseudotime irrespective of Th1/Tfh state and therefore interpreted as being 'associated with memory onset'. How

then do the authors interpret their flow cytometry data showing that Id2 levels are highest on day 7, the peak of the effector response, so much earlier than the onset of memory, and that during memory (d28), the expression levels are lower again (Fig 4E)? To me the flow data do not support the conclusion that Id2 expression is associated/correlated with memory onset. What might also help is a clear description of how the authors define 'memory onset'. Similarly, cMaf (Fig 4F) is expressed at higher levels at day 7 than 28 – to me this does not argue for a 'role for cMaf in CD4 memory in malaria'.

We apologise for the confusion, due perhaps to lack of clarity in our reasoning and definitions. We propose that partial retention of gene expression at day 28, at levels higher than those in naive cells implicated a gene in memory onset or maintenance. Whether or not a gene was highly expressed at effector stages was less relevant in our reasoning. We have amended our text (Lines 305-311, see below) and figures to focus on Naive vs Day 28 assessments for Id2, Tcf1 and c-Maf. We present multiple independent experiments (n=4, with 5-6 mice per group), which show that these transcription factors are elevated at protein level in memory. Given this, we propose these TFs may play a role in some aspect of memory, either preservation or onset.

(Lines 305-311): *"To validate predictions from our probabilistic models, we examined protein expression of various memory-associated markers, including their distribution amongst Th1- or Tfh-lineages (Supplementary Figure 6a). We confirmed intracellular Id2 protein upregulation in all PbTILs at day 28 p.i., and a subset expressing the reciprocally-related protein{Masson, 2013 #7154}, TCF1 (encoded by Tcf7), the proportion of which increased with IAT (Supplementary Figure 6b). We observed intracellular cMaf in all PbTILs at day 28 p.i., in the presence or absence of IAT, (Supplementary 6c), suggesting a possible role for this transcription factor in CD4⁺ T cell memory in malaria."*

In relation to this, in Figure 4E the gates are very different for day 7 than all other days, which means that comparing the resulting cell frequencies between day 7 and the other days is somewhat misleading. Given that these transcription factors do not identify clear + and – populations, but rather present a shift in mean expression levels, I would find it more appropriate to evaluate the shifts in Id2, TCF1 and cMaf expression (4E+F) by (geo)MFI, rather than by % positives. The same applies to Fig 5G.

The Day 7 data is surplus to requirements in this figure (and was not specifically referred to in the text of the original submission). It has therefore been removed to allow focus on day 28 data. As requested, we have adjusted previous Figure 4e and 4f (now presented in Supplementary Fig 6b & c) and in Figure 5g to include both MFI metrics. These are presented below for reference. Account of the data in the Results section does not need to be modified since we had not referred to Day 7 data in the original submission.

Supplementary Figure 6b-c (previously Fig 4e+f)

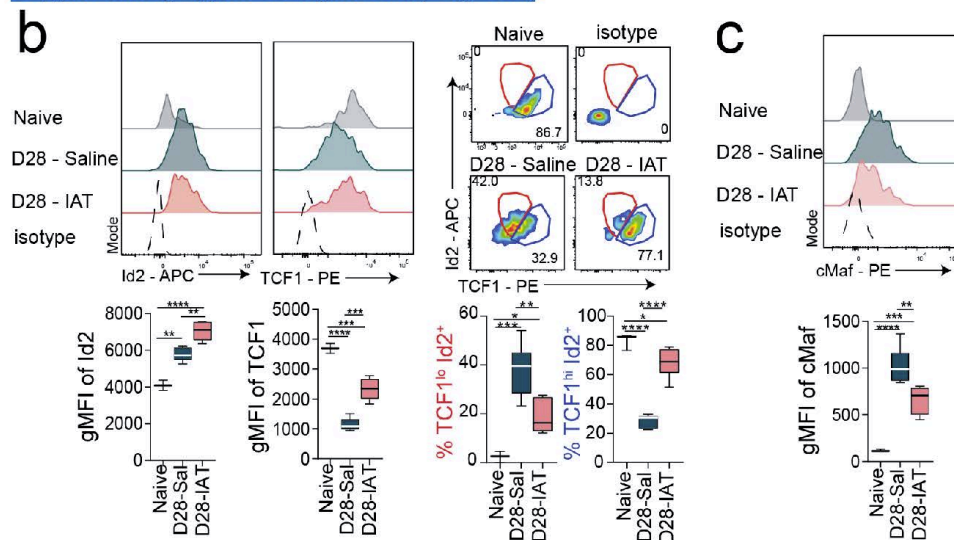
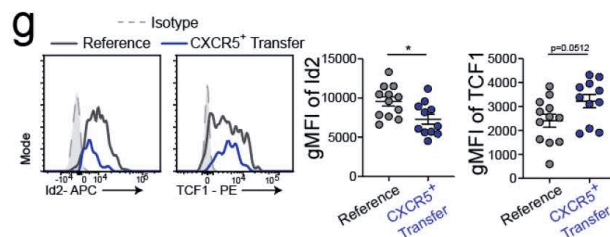


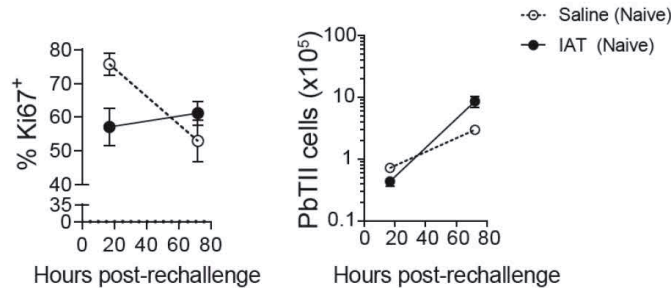
Figure 5g



S2E+F: How do the authors interpret their finding that activation of naïve T cells leads to a lower % of Ki67+ cells in the presence of IAT than saline. Does IAT reduce responses of naïve T cells? Is that a desirable effect of the drug?

The difference referred to, observed at 18 hours post-challenge was modest and transient, since similar assessment at 72 hours in the same experiment revealed a loss of this effect. We present the data below for the Reviewer's benefit, showing the % of PbTII cells expressing Ki67, and the total number of PbTII cells derived from these naïve cells at 18 and 72 hours post-rechallenge. Both data show the effect to be transient. The modest differences seen in the initial response of naïve PbTII cells under these two conditions could be considered alongside the dramatic differences in spleen microarchitecture and splenomegaly that exist. We speculate that priming and early activation of naïve PbTII cells is likely more effective in IAT-treated mice, where splenomegaly and negative consequences of persisting infection have been minimised. Since the naïve cells were

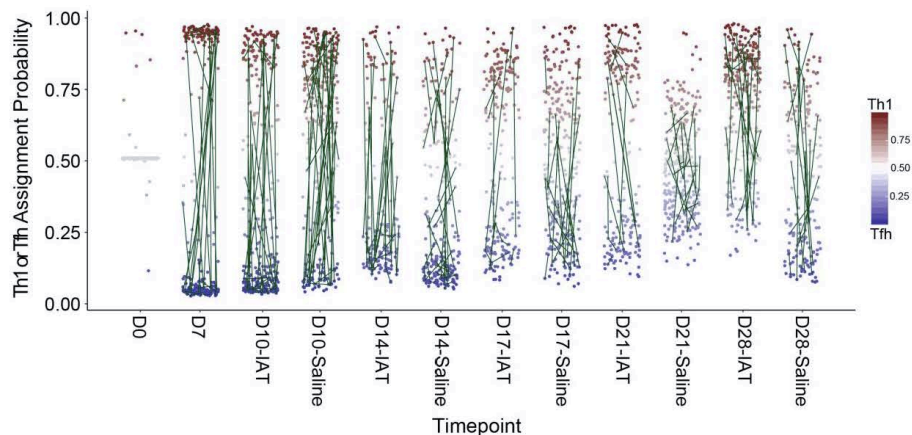
incorporated in the study only as comparators, we do not propose including the data below in the study, since it may detract from our desired focus on memory PbTILs in Supp Figure 2.



The clonal analysis based on endogenous TCRab sequences is a beautiful system, but also comes with caveats. The authors mention that 'as expected, family members did not cross timepoints or conditions (data not shown)'. I believe this is a very crucial control if one wants to conclude on clonal overlap between fates. Therefore, I would pledge for the data of this control to be shown.

We acknowledge that confirmation of the fidelity of our approach is important. Therefore, we present new Supplementary Figure 7a, also shown below for reference, the proof that siblings do not cross time-points or conditions. In Figure S7a, green lines indicate wherever in the dataset, sibling pairs are detected. Please note that green lines NEVER cross from one timepoint/condition to another.

S7a

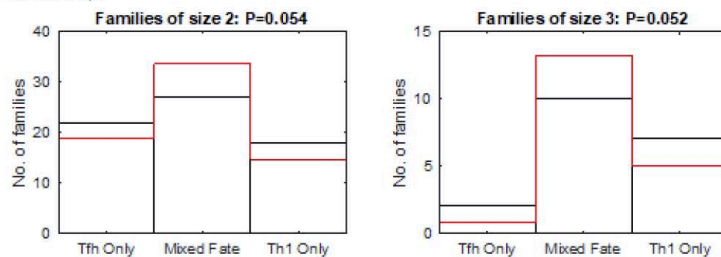


Since the chances that a single family has detectable members in both the Th1 and Tfh fate is higher when the number of detected families is higher, I would like to see

whether the authors' conclusion with regards to Fig 5D holds true if only families are depicted that have an equal number of detected members. So, for example when only those families are plotted that have two detected members (and only those that have 3 members – if these are enough families to assess).

We have undertaken the requested analysis. When the same analysis is performed as in figure 5B but with only families of size 2 ($n=67$) and 3 ($n=19$), respectively, we find the same pattern of over representation of homogenous families (Figure below). These two trends (each showing marginal significance due to less power with a lower number of families, $P=0.054$ and $P=0.052$, respectively), reaffirm the significant result observed when analysis is performed across all families. Given this outcome, we feel the original findings remain valid, and propose not to alter the depiction or interpretation of the data. We have included the following text in the Results to deal with this issue (Lines 349-352):

"Instead, PbTfIs from the same family exhibited a greater tendency towards the same fate than predicted by a random binomial process ($p<0.0005$, Figure 5d), the trend being evident also during examination of smaller families, albeit with reduced statistical significance due to smaller sample sizes ($p=0.054$ for 67 families of two; $p=0.052$ for 19 families of three)."



To address whether 'Th1 or Tfh effectors surviving contraction remained on their apparent Th1 or Tfh developmental trajectories with no lineage plasticity', the authors track the fate of adoptively transferred d7 sorted Tfh and Th1. The subsets are transferred into mice receiving IAT, and not into mice receiving saline, because, as the authors state, in the IAT group 'apparent developmental trajectories [inferred from scRNAseq data] were better preserved over time'. Exactly for that reason, I think it is crucial to transfer the subsets (additionally) into the saline group recipients, to avoid any potential selection bias. The cells transferred into the IAT treated mice partially retained their respective Th1/Tfh phenotype, but is this just because they were exposed to IAT? Or does this also happen in the 'natural' untreated setting? The scRNAseq data in the saline group show more convergence of Tfh/Th1 RNA profiles towards each other at later pseudotime. Is this indicative of fate plasticity in the absence of IAT? Does IAT limit plasticity? This can be addressed by adoptively transferring the cells into mice that will not be treated with IAT vs those that will be treated. I think such an experiment would strengthen the manuscript substantially.

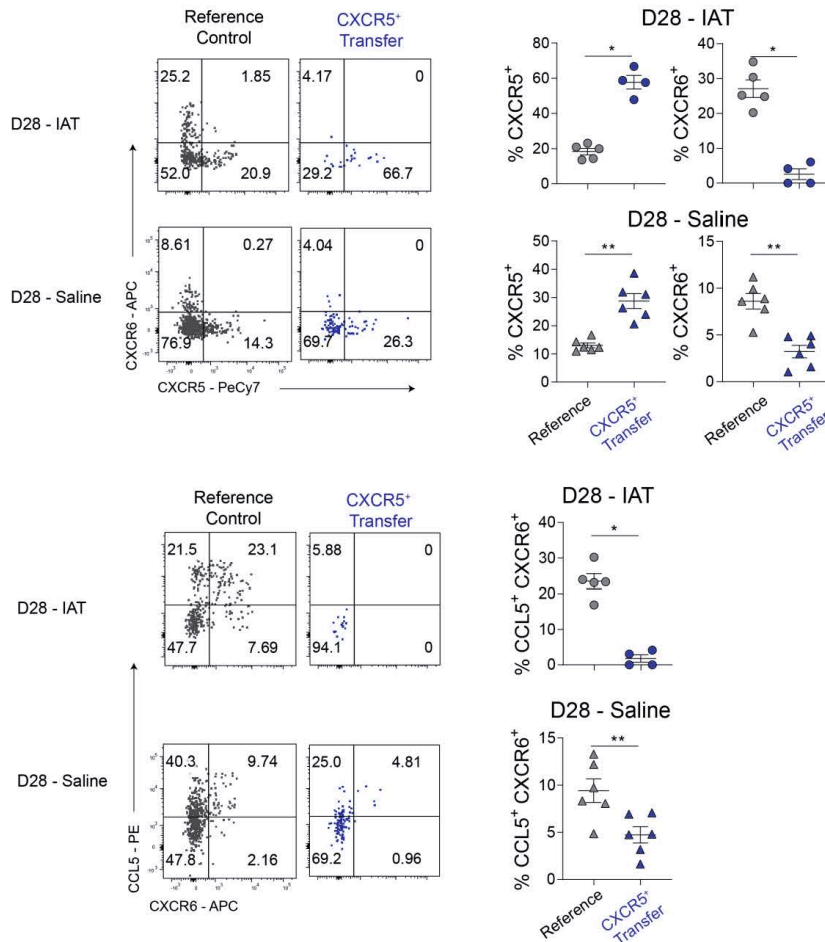
Despite COVID19 restrictions on wet-lab research at our Institutes, we were able to conduct this experiment. We CD4-MACS-enriched cells from 40 spleens of Day 7 *PcAS*-infected mice, cell-sorted both CXCR5⁺ (“Tfh/Tcm lineage”) and CXCR6⁺ (“Th1/Tem lineage”) effector PbTILs, and transferred ~250,000 separately into infection-matched recipient mice. Importantly, we compared these to a “reference” group, which had received 10,000 naive PbTIL cells on day -1, *PcAS* infection on Day 0, with no further cell transfers made. As before, we treated the three groups, “CXCR5⁺ transfer”, “CXCR6⁺ transfer” and “Reference”, with IAT or control Saline from Day 7 until Day 27. On Day 28, spleens were assessed for the phenotype of PbTILs (now presented in Supplementary Figure 7b & c, also shown below for reference).

As in the original manuscript, under IAT treatment CXCR5⁺ “Tfh/Tcm lineage” effectors once again did not transition to a Th1-memory phenotype, as assessed by the top Th1-associated marker, CXCR6, as well as the subsidiary intracellular Th1 associated marker, CCL5. Crucially, we now show in non-drug treated mice, CXCR5⁺ effectors were again poor at transitioning to a Th1-phenotype, consistent with a lack of cross over between trajectories. Therefore, during persisting infection or IAT treatment, PbTILs progressing along the Tfh/Tcm trajectory in the spleen do not readily cross over to the Th1 lineage. This new data cements our view that trajectories inferred from computational modelling of scRNA-seq data appear to be real.

(As in the original experiments (already discussed in the original manuscript), we found it hard to locate cells derived from CXCR6⁺ effectors in the spleen, although they were detected as CXCR6⁺ CXCR5⁺ cells in the liver. We continue to propose highly activated Th1 effectors, expressing numerous chemokine receptors, are more prone to altered trafficking once removed and re-injected intravenously compared to CXCR5⁺ counterparts. This does not interfere with our conclusions.)

We include the following text in the Results to relate our new findings (Lines 370-372):

“Fate-mapping also performed during persisting infection elicited a similar outcome, suggesting that IAT treatment itself had not restricted lineage plasticity (Supplementary Figure 7b & c).”



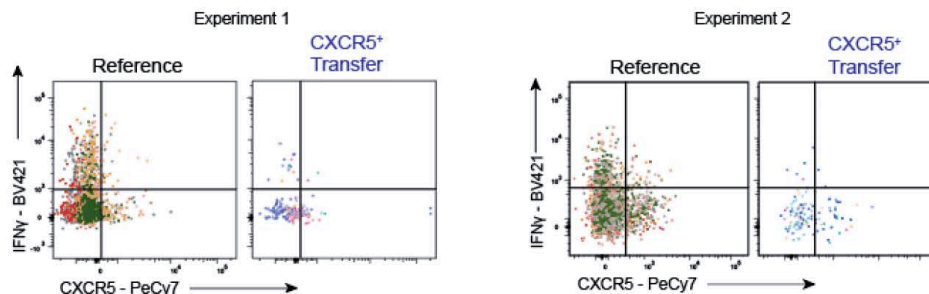
How do the authors explain the loss of CXCR5 expression on most of the sorted CXCR5+ cells after transfer? Did most of the transferred Tfh convert into an undefinable state, while only a minority (those that retained CXCR5) remained faithful to their Tfh fate? In that regard, the IFN γ production is quite variable in the CXCR5+ transfer group. Can this be explained by analyzing a mixture of 'true' Tfh that kept their Tfh fate, mixed with some undefined other CD4 state? How is IFN γ production if the CXCR5+ transfer group is stratified into those cells that kept their CXCR5 expression and those that lost it?

Our scRNA-seq modelling suggests CXCR5 down-regulation is a general feature of cells within the Tfh/Tcm trajectory. However, heterogeneity in CXCR5 retention was evident within transferred CXCR5+ effectors. We feel it is beyond the scope of this current study

to examine this heterogeneity dynamically. Nevertheless, our Day 28 droplet-based scRNA-seq data is consistent with trifurcation of CXCR5⁺ effectors into GC Tfh, memory Tfh and Tcm cells. Future projects will decipher possible branching events within the Tfh/Tcm trajectory during malaria. To reflect this point, we include the text below in the Discussion (Lines 581-585).

“Interestingly, many cells derived from CXCR5⁺ effectors lost CXCR5 expression while a minority preserved it. Although, scRNA-seq modelling suggests that CXCR5 down-regulation is a general feature of cells in the Tfh/Tcm lineage, our study did not explore possible heterogeneity in this response. Future studies could explore heterogeneous trajectories in CXCR5⁺ effectors that lead to Tfh versus Tcm phenotypes.”

In answer to the Reviewer's specific request, we present for the Reviewer's benefit (not in the manuscript) the analysis of *in vitro* PMA/ionomycin re-stimulated IFN γ production by all PbTILs derived from CXCR5⁺ effectors. Due to small numbers of PbTILs recovered from each mouse, we present concatenated FACS plots (coloured by mouse) for two independent experiments. IFN γ is mostly produced by cells with low/no CXCR5 expression, but cell numbers are not sufficient to test this statistically. Importantly, cells derived from CXCR5⁺ effectors exhibited lower IFN γ production *in vitro* compared to IFN γ producing cells in reference controls. Our data is consistent with more than one cell-type existing amongst cells derived from CXCR5⁺ effectors.



In 6F, please show the gate that defines whether a cell is considered Tigit⁺ or –
This is now shown on the graph.

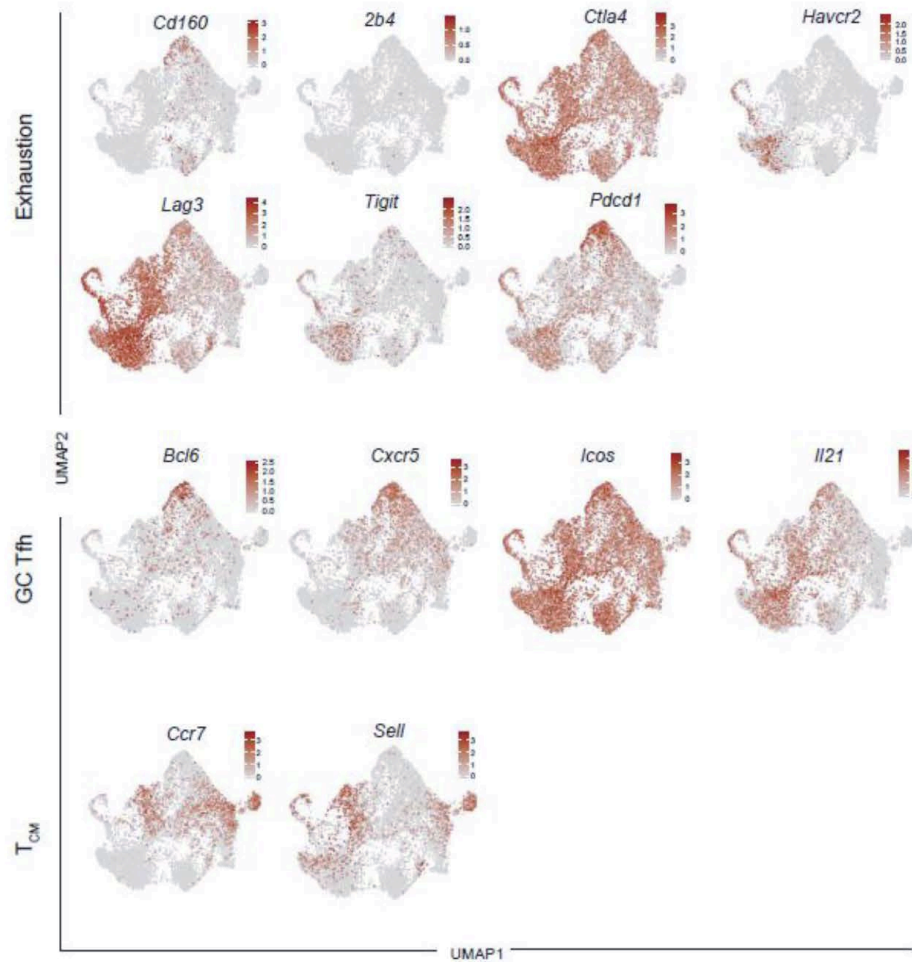
6C: What is cluster 8? Please comment

This is now inserted in the manuscript in line 433: *“Cluster 8 (characterised by a Type I IFN-associated signature)”*

6E/G: For completeness I would like to see the scores for all clusters, and the expression patterns (like in 6D) of the individual genes making up the scores, in particular if the scores are just made up of a few genes (like Tcm)

Scores for all clusters are now shown in Figure 6e & 6g.

In addition, we present below for the Reviewer's benefit (not in the manuscript), the expression patterns for genes making up each score. Please note that *Pdcd1* is common to both the exhaustion and GC Tfh signatures, though shown here as part of exhaustion only.



Row 419: 'IAT had significantly improved the quality of ...memory responses...by boosting Tcm and CG Tfh responses'. Why is it better for the quality of the response to boost Tcm / CG Tfh responses? In order to make the strong point that IAT improves the quality of the memory response, a functional measurement of quality would be

warranted. Are mice better protected from a secondary malaria infection if they were treated with IAT vs saline after the primary infection?

We agree that ideally, functional measurement of improved immunity should be conducted when possible. Based on our previous studies of Type I IFN-mediated immune-suppression of Tfh responses and antibody production in malaria (Sebina I *et al*, *Plos Pathog* 2016, James KR *et al J Immunol* 2018) we reasoned that increased GC Tfh proportions in particular may be beneficial for eliciting immunity to re-challenge.

Unfortunately, while the *P. chabaudi* AS model is useful for studying the generation and regulation of cellular and humoral immunity during primary infection, the “strength” of immunity to rechallenge is so robust in wild-type, un-manipulated mice (which control at least a 1000-fold increase in infectious dose with almost no resulting parasitemia compared to primary infection, Akter J. *et al (PloS Pathog* 2019)) that the system can not be used to search for improvements in parasite control during re-challenge - we tried unsuccessfully to establish this approach for a recent study on IRF3, James KR *et al J Immunol* 2018.

This caveat has been raised in the Discussion (Lines 671-679), also shown below:

“A caveat of our murine system is that immunity to homologous re-challenge elicited by primary infection, is so robust that improvements to parasite control cannot be assessed functionally{Akter, 2019 #7200}. Nevertheless, we previously correlated elevated Tfh responses with improved primary immunity to malaria in mice{Sebina, 2016 #6407;Sebina, 2017 #204;James, 2018 #381}. We speculate that immunological processes such as T cell exhaustion, impaired antigen presentation via Type I IFN-signalling {Montes de Oca, 2016 #7208;Haque, 2014 #5425}, and immune-suppression via IL-10 {Plebanski, 1999 #615;Saraiva, 2009 #371;Jagannathan, 2014 #163} {Jagannathan, 2016 #6402;Couper, 2008 #3725}. {Matar, 2015 #616}all serve to impair GC Tfh responses, and are alleviated by lowering parasite burden with anti-malarial drugs. In endemic areas, individuals, often children, are likely infected multiple times each season. The effect of multiple infections and rounds of IAT on CD4⁺ T cells remains to be tested in vivo”

Patterns of transcriptional and epigenetic changes from naïve to effector to memory states are quite well described for CD8 T cells (e.g by groups of Ben Youngblood, Ananda Goldrath, Rafi Ahmed). These studies also found that numerous epigenetic changes that take place after T cell priming are retained from effector to memory phase. I would find it appropriate to discuss these.

We acknowledge the importance of relating our work to studies of memory CD8⁺ T cells. We have now included comment on CD8⁺ T cells in the Discussion (Lines 654-665), also reproduced below:

“Several studies have examined epigenomic and transcriptomic changes in CD8⁺ T cells during memory development, mostly employing LCMV or Listeria infection in mice {Scott-

Browne, 2016 #425;Kakaradov, 2017 #423;Youngblood, 2017 #523;Yu, 2017 #525}, or in humans undergoing vaccination, or cancer treatment{Akondy, 2017 #526;Li, 2019 #809;Satpathy, 2019 #810}. These studies variously assessed chromatin accessibility, histone-modification, DNA methylation, chromosome conformation and gene expression, and provided support in CD8⁺ T cells for effector to memory transitions accompanied by partial, though incomplete, reversion to naive-like states. This prevailing CD8⁺ T cell model is largely consistent with that presented here for CD4⁺ T cells during experimental malaria. Although there may be differences between aspects of CD8⁺ T cell differentiation and that of CD4⁺ T cells, such as early bifurcation into short-lived effectors versus memory precursors, or the existence of a stem-like state in human CD8⁺ T cells, we nevertheless propose fate bifurcation towards multiple effector-like states, followed by partial reversion to a naive-like state in memory as a general feature of conventional TCRαβ T cells.”

The authors suggest that anti-malarial drug treatment of humans in endemic regions might serve to support the generation of parasite-specific CD4 memory. Their experiments investigated the effect of anti-malarial drug treatment after primary infection. In endemic regions humans are usually infected for the first time at an early age and will be infected much more often during the course of their life. Therefore, for endemic regions it is much more relevant to know whether anti-malarial treatment affects memory generation after repeated malaria exposures, or only after the first exposure. This could be experimentally addressed by evaluating the memory T cells generated after repeated malaria exposures, with IAT administered after a secondary response.

We agree that in theory, multiple rounds of infection/IAT could be conducted in mouse models. However, we propose the approach is beyond the scope of this study. We have included the following comment on this topic in the Discussion, at lines 677-679.

“In endemic areas, individuals, often children, are likely infected multiple times each season. The effect of multiple infections and rounds of IAT on CD4⁺ T cells remains to be tested in vivo.”

When discussing their finding that single naïve CD4 can give rise to both Tfh and Th1 fates, but not necessarily always produce both fates, I would find it appropriate to discuss that an earlier study has come to the same conclusion in another infection model (Marc Jenkins lab, Tubo et al).

We agree, and have ensured this point is clearly made in the Discussion, Lines 604-609, also shown below:

“Fitting to a mathematical model revealed a modest pre-disposition to either one fate or the other within families derived from the same naïve cell. This suggested modest heterogeneity in responses of individual clones, despite transcriptomic and epigenomic homogeneity in naivety, and predictable frequencies of each fate at a population level. Our observations are entirely consistent with a previous report, which used bacterial infection and single-cell adoptive transfer of TCR transgenic CD4⁺ T cells{Tubo, 2013 #5422}.

When discussing TCF-1 expression in the Tfh lineage, it would be relevant to discuss parallels to CD8 T cells, since their model of persistent malaria infection results in a certain degree of T cell exhaustion, and since TCF-1 plays a crucial role in CD8 differentiation in the context of persistent viral infections (e.g. LCMV clone 13) which also leads to exhaustion (e.g. groups of John Wherry, Dietmar Zehn, Rafi Ahmed)

We acknowledge this point, and have inserted new text in the Discussion. The text is present at Lines 634-641, also shown below:

"These observations are reminiscent of those in CD8⁺ T cells, where TCF1 supports the generation both of antigen-experienced, stem-like memory cells in humans, that exhibit a capacity for self-renewal, proliferation and plasticity, as well as progenitors of exhausted cells in mice and humans that can be re-invigorated via immune checkpoint blockade {Siddiqui, 2019 #815; Chen, 2019 #816}. These observations, in addition to others {Nish, 2017 #7057; Lin, 2016 #7161}, and our recent description of TCF1⁺ allo-reactive CD4⁺ T cells in the gut {Engel, 2020 #807}, suggest a generalised T cell model, in which TCF1 is retained along one developmental branch after fate bifurcation, supporting the potential of some memory T cells to mount effector or memory responses at a later time."

End of Response to Reviewers.

Decision Letter, first revision:**Subject:** Decision on Nature Immunology submission NI-RS29275A**Message:** Dear Dr Haque,

Thank you for your response to the reviewers' comments on your manuscript "Transcriptome Dynamics of CD4⁺ T cells during Malaria Maps Progressive Transition from Effector to Memory.". We are happy to inform you that if you revise your manuscript appropriately in response to the referees' comments and our editorial requirements your manuscript should be publishable in Nature Immunology.

Please revise your manuscript according with the reviewers' comments and as outlined in your letter. At resubmission, please include a point-by-point response to the referees' comments, noting the pages and lines where the changes can be found in the revision. Please highlight the changes in the revised manuscript as well.

We are trying to improve the quality and transparency of methods and statistics reporting in our papers (please see our editorial in the May 2013 issue). Please update the Life Sciences Reporting Summary, and supplements if applicable, with any information relevant to any new experiments and upload it (as a Related Manuscript File) along with the files for your revision. If nothing in the checklist has changed, please upload the current version again.

TRANSPARENT PEER REVIEW

Nature Immunology offers a transparent peer review option for new original research manuscripts submitted from 1st December 2019. We encourage increased transparency in peer review by publishing the reviewer comments, author rebuttal letters and editorial decision letters if the authors agree. Such peer review material is made available as a supplementary peer review file. **Please state in the cover letter 'I wish to participate in transparent peer review' if you want to opt in, or 'I do not wish to participate in transparent peer review' if you don't.** Failure to state your preference will result in delays in accepting your manuscript for publication.

Please note: we allow redactions to authors' rebuttal and reviewer comments in the interest of confidentiality. If you are concerned about the release of confidential data, please let us know specifically what information you would like to have removed. Please note that we cannot incorporate redactions for any other reasons. Reviewer names will be published in the peer review files if the reviewer signed the comments to authors, or if reviewers explicitly agree to release their name. For more information, please refer to our <https://www.nature.com/documents/nr-transparent-peer-review.pdf> FAQ page.

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We hope to receive your revised manuscript in 10 days, by 4th Aug 2020. Please let us know if circumstances will delay submission beyond this time. If you have any questions please do not hesitate to contact me.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
Tel: 212-726-9207
Fax: 212-696-9752

z.fehervari@nature.com

Reviewer #2 (Remarks to the Author):

The revised version of the manuscript is much improved over the previous version, with provision of additional experiments, computational analyses and appropriate discussions and references. I believe the huge amount of sequencing data from multiple timepoints plus the GUI to explore the data are a valuable resource to the community that functions as a valuable hypothesis generator.

Most of my previous concerns have been adequately addressed. Two points however still remain, and a third one has come up in relation to the newly presented data.

1)

My previous point still holds, that the presented data do not support the conclusion that the transcription factors Id2 and cMaf are associated with memory. The authors model based on the transcriptional data identifies Id2 and cMaf as a transcription factors associated with late pseudotime irrespective of Th1/Tfh state. This hypothesis is however not supported by the presented protein data on these two transcription factors. In the earlier version of the manuscript, the authors presented flow cytometry data for naïve, d7 (effector) and d28 (memory) cells, showing that both transcription factors are expressed in both effector and memory cells, and in fact even at higher levels in the effector cells than the memory cells. This shows that expression of both transcription factors is associated with antigen-experienced cells (i.e. both effector and memory cells), and not just with memory cells. In the current version of the manuscript the authors chose to not show the d7 data, but still draw the same conclusion.

'suggesting a possible role for this transcription factor in CD4+ T cell memory in malaria', 'our transcriptomic model correctly predicted the expression and distribution of several memory-associated transcription factors', 'We also defined a small subset of genes associated with memory onset in both lineages, including the transcription factors Id2 and Maf...'

In order to judge whether molecules are associated with memory, it is however crucial to not just memory and naïve cells, but also effector cells, since indeed when expression occurs in both effector and memory phases, the molecule cannot be considered associated with memory alone, but with all antigen experienced cells. I suggest that the authors 1) either clearly state that although their computational model predicts that Id2 and cMaf are associated with memory (irrespective of Th1/Tfh), the protein data do not validate these findings and instead show that both are associated with both effector and memory cells (and also show the now omitted d7 data), or 2) that the authors refrain from discussing these two molecules and instead focus the analysis & validation on the comparison of the Th1 vs Tfh comparison at the memory timepoint, and the saline vs IAT condition at the memory timepoint, which are highly valuable comparisons.

2)

I am still concerned about the CCR7 stain presented in 1d and 2e. As I mentioned in my previous comment on this, the currently commercially available antibodies (including the one the authors used) are quite famous for not being able to give a very good resolution. A separation between naïve T cells (which express relatively high levels of CCR7) and CCR7- cells already requires adjustments to standard staining protocols, where staining time and/or temperature are significantly increased to see a separation. The manufacturer of the antibody the authors used demonstrates this quite nicely in their product data sheet

supplement:

See: <https://www.biolegend.com/en-us/products/pe-anti-mouse-cd197-ccr7-antibody-2799>

'Staining with clone 4B12 is recommended at 37°C (see supplemental data of PE staining at differing temperatures)' with staining times of 15-20 min according to Biolegend. I cannot insert the pdf here - click on '(see supplemental data of PE staining at differing temperatures)' to download the FACS data.

Also papers that have examined CCR7 CD4 T cells responding to infections and managed to see an OK separation between CCR7⁻ and CCR7 high naïve T cells only managed to see this level of CCR7 resolution after staining for e.g. 1h at room temperature (e.g ref 9 of manuscript). That these published data come from a different infection model cannot explain the difference in CCR7 level detection between CCR7⁻ and CCR7 high naïve T cells. In this context, and given that the authors report to have performed stainings at 4°C and for just 20min, the extremely good resolution/separation of CCR7⁺ vs CCR7⁻ cells AMONG memory T cells in Figure 1d seems to me too good to be true. How do the authors explain the extreme discrepancy in CCR7 staining resolution between their data and other published data with the same antibody, and data from the manufacturer of their antibody? Is there any chance that for example compensation is not optimal?

In relation to this, the authors' CCR7 stain presented in S8d has not such a good resolution as in 1d, while both experiments show CCR7 staining on d28 transferred CD4 T cells in spleen after IAT. How do the authors explain this difference? In the S8d experiment it would be helpful to add the CCR7 staining level of the endogenous cells, which includes both effector and naïve cells with the highest CCR7 levels and thereby provide a good range of the detectable staining intensities.

3)

In the new figure S8d the authors compare CCR7 expression on memory cells between spleen and LN. However, the cutoff for what is considered + vs - in both organs is not placed at the same CCR7 level. Cells with lower CCR7 level are scored as + in the LN than spleen, which will contribute to having a higher % of CCR7⁺ cells in LN than spleen in the graph to the right.

The peak of the isotype control seems at the same spot in both organs, so the control is fine and the same cutoff for CCR7⁺ vs - needs to be applied. It seems that the cutoff was placed at the right end of the isotype distribution in both organs, which is misleading because more cells make up the isotype peak in the spleen than LN - this of course makes the distribution wider and should therefore not be used to define a cutoff.

Additional minor points

1) In the new figure S7c it visually seems that the difference between the reference and transfer groups is larger with IAT than saline, nevertheless the * suggest the opposite. Is the visual impression misleading, or is there a chance the * have been accidentally swapped?

2) According to the methods section, 'To assess cytokine and transcription factor expression, cells were incubated with brefeldin-A (10 mg/ml) with or without ionomycin (500 ng/ml) and PMA...'. Is this a typo? Or were transcription factors indeed assessed after stimulation with PMA/ionomycin? If so, this would be quite unconventional and should

therefore be explicitly mentioned in the text, since it would significantly influence the interpretation of the transcription factor data – the stimulation itself may have influenced the expression of the transcription factor and might therefore not at all represent the state of the cells at isolation from the tissue.

Author Rebuttal, first revision:

We are grateful for the opportunity to modify our manuscript in response to minor concerns raised by Reviewer #2, and in accordance with our proposed plan of action outlined to Zoltan Fehervari. In our recent proposal we stated the following:

"In summary, for the main three concerns, we propose to:

- (1) Modify the manuscript and figure relating to cMaf and Id2 according to Reviewer #2's "Option 1".*
- (2) Correct an omission from our methods, to show we indeed stained for CCR7 correctly, at 37°C for 1 hour.*
- (3) To remove the lymph node CCR7 staining data from Figure S8d, since it is surplus to requirements for examining Th1 phenotypes."*

We present below a point-by-point response to Reviewer 2:

Reviewer #2

(Remarks to the Author)

The revised version of the manuscript is much improved over the previous version, with provision of additional experiments, computational analyses and appropriate discussions and references. I believe the huge amount of sequencing data from multiple timepoints plus the GUI to explore the data are a valuable resource to the community that functions as a valuable hypothesis generator. Most of my previous concerns have been adequately addressed. Two points however still remain, and a third one has come up in relation to the newly presented data.

1)

My previous point still holds, that the presented data do not support the conclusion that the transcription factors Id2 and cMaf are associated with memory. The authors model based on the transcriptional data identifies Id2 and cMaf as transcription factors associated with late pseudotime irrespective of Th1/Tfh state. This hypothesis is however not supported by the presented protein data on these two transcription factors. In the earlier version of the manuscript, the authors presented flow cytometry data for naïve, d7 (effector) and d28 (memory) cells, showing that both transcription factors are expressed in both effector and memory cells, and in fact even at higher levels in the effector cells than the memory cells. This shows that expression of both transcription factors is associated with antigen-experienced cells (i.e. both effector and memory cells), and not just with memory cells. In the current version of the manuscript the authors chose to not show the d7 data, but still draw the same conclusion.

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In order to judge whether molecules are associated with memory, it is however crucial to not just memory and naïve cells, but also effector cells, since indeed when expression occurs in both effector and memory phases, the molecule cannot be considered associated with memory alone, but with all antigen experienced cells. I suggest that the authors 1) either clearly state that although their computational model predicts that Id2 and cMaf are associated with memory (irrespective of Th1/Tfh), the protein data do not validate these findings and instead show that both are associated with both effector and memory cells (and also show the now omitted d7 data), or 2) that the authors refrain from discussing these two molecules and instead focus the analysis & validation on the comparison of the Th1

vs Tfh comparison at the memory timepoint, and the saline vs IAT condition at the memory timepoint, which are highly valuable comparisons.

We have modified the manuscript according to “Option 1” from the Reviewer, which re-introduces Day 7 data back into the Supp Figure 6, allowing to reader to see the entire dataset. Moreover, we now clearly state that Id2 and cMaf are most strongly expressed at the effector stage, and that while Id2 is not retained at protein level during transit to memory, cMaf protein is partially retained at a low level.

This is now amended in the Results text (lines 305 -318):

“To test predictions from our probabilistic models (Supplementary Figure 6a), we examined protein expression of various effector- and memory-associated markers, including their distribution amongst Th1- or Tfh-lineages. Intracellular Id2 was most strongly upregulated in effectors at day 7 p.i., but was not substantially retained at protein level in memory cells by day 28 p.i. (Supplementary Figure 6b). Reciprocally-related TCF1 (encoded by Tcf7)⁴³, was heterogeneously downregulated in effectors compared to naïve cells, and was partly recovered in a subset of memory cells during IAT (Supplementary Figure 6b). We noted strong intracellular cMaf upregulation in all effector PbTILs at day 7 p.i., which was partially retained at day 28 p.i. in the presence or absence of IAT (Supplementary 6c). We tested predictions that CCL5 was associated with Th1 effector and memory cells, while CXCR3 was universally expressed (Supplementary Figure 6d & e). Consistent with this, direct ex vivo intracellular expression of CCL5 was absent from effector and memory CXCR5⁺ PbTILs, but was associated with CXCR6⁺ memory PbTILs (Supplementary Figure 6d), whereas CXCR3 was broadly expressed on most PbTILs, including CXCR5⁺ and CXCR6⁺ cells at day 28 p.i. in the presence of IAT (Supplementary Figure 6e).”

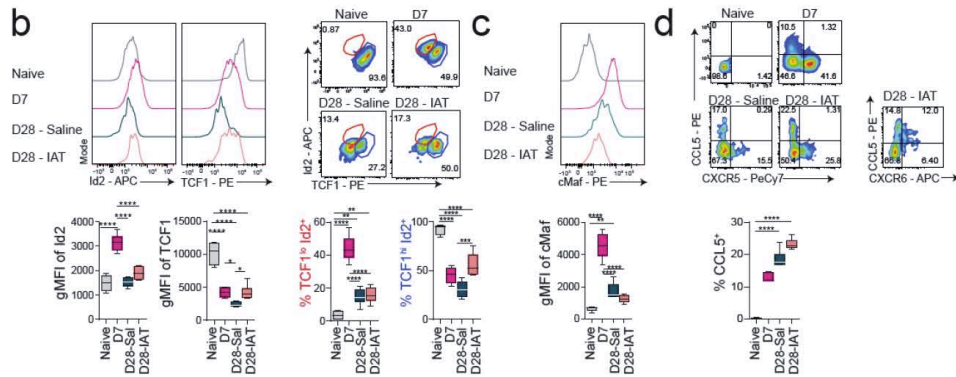
And in the Discussion in lines 641-644:

“We also defined a small subset of genes associated with effector to memory transit, including the transcription factor Maf, chemokine-related genes, Ccl5 and Cxcr3, integrin Itgb1, and cytokine receptor Il2rb, some of which were validated at protein level. We hypothesize this gene set may be useful for distinguishing naïve from memory states in polyclonal CD4⁺ T cell populations, particularly after Th1-biased immune-challenges.”

And, lines 650-653:

“For example, Maf and Irf8 motifs were retained in memory cells relative to naïve; given expression of Maf during effector to memory transit, and low-level retention of intracellular cMaf protein, we hypothesize it could play a functional role in memory CD4⁺ T cells.”

The amended Results figure is also shown here:



2)

I am still concerned about the CCR7 stain presented in 1d and 2e. As I mentioned in my previous comment on this, the currently commercially available antibodies (including the one the authors used) are quite famous for not being able to give a very good resolution. A separation between naïve T cells (which express relatively high levels of CCR7) and CCR7⁻ cells already requires adjustments to standard staining protocols, where staining time and/or temperature are significantly increased to see a separation. The manufacturer of the antibody the authors used demonstrates this quite nicely in their product data sheet supplement:

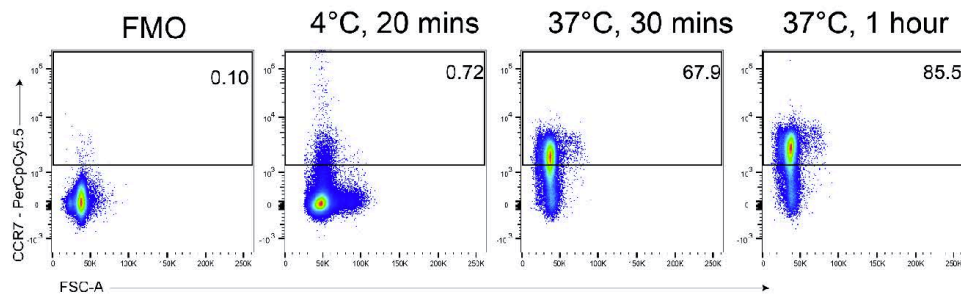
See: <https://www.biologend.com/en-us/products/pe-anti-mouse-cd197-ccr7-antibody-2799>

'Staining with clone 4B12 is recommended at 37°C (see supplemental data of PE staining at differing temperatures)' with staining times of 15-20 min according to Biologend. I cannot insert the pdf here - click on '(see supplemental data of PE staining at differing temperatures)' to download the FACS data.

Also papers that have examined CCR7 CD4 T cells responding to infections and managed to see an OK separation between CCR7⁺ and CCR7⁻ high naïve T cells only managed to see this level of CCR7 resolution after staining for e.g. 1h at room temperature (e.g ref 9 of manuscript). That these published data come from a different infection model cannot explain the difference in CCR7 level detection between CCR7⁻ and CCR7⁺ high naïve T cells.

In this context, and given that the authors report to have performed stainings at 4°C and for just 20min, the extremely good resolution/separation of CCR7⁺ vs CCR7⁻ cells AMONG memory T cells in Figure 1d seems to me too good to be true. How do the authors explain the extreme discrepancy in CCR7 staining resolution between their data and other published data with the same antibody, and data from the manufacturer of their antibody? Is there any chance that for example compensation is not optimal?

We apologise for the mis-leading omission from our methods of the specific approach we employed for CCR7 staining, because we did indeed follow the manufacturer's instructions to stain at 37°C for 1 hour. Below is an example of our early CCR7-staining optimisation, comparing staining at 4°C for 20 mins versus 37°C for 30 minutes and 1 hour (see below). Our experience is therefore consistent with the Reviewer's comments and manufacturer's guidelines, with all CCR7-staining data in the manuscript produced with the appropriate protocol.



Samples are naive CD4⁺ T cells

We have now corrected this in our materials and methods (line 1032-1033):

"Staining for CCR7 surface antibody was performed at 37°C for 1 hour as per manufacturer's recommendation, after Fc receptor blocking."

In relation to this, the authors' CCR7 stain presented in S8d has not such a good resolution as in 1d, while both experiments show CCR7 staining on d28 transferred CD4 T cells in spleen after IAT. How do the authors explain this difference? In the S8d experiment it would be helpful to add the CCR7 staining level of the endogenous cells, which includes both effector and naïve cells with the highest CCR7 levels and thereby provide a good range of the detectable staining intensities.

As suggested by the Reviewer's comments and manufacturer's instructions, CCR7 staining may be more sensitive than most conventional antibody stains, and may indeed vary from experiment to experiment. We think this accounts for the differences identified by the reviewer. However, because of the Reviewer's point below regarding the modest differences in CCR7 staining between spleen and LN, we removed the CCR7 data from S8d. Most importantly, this data is not needed to confirm the Reviewer's hypothesis that Th1-memory cells are restricted from lymph node.

This data has now been removed (lines 386-388):

"Consistent with this, paired analysis across tissues revealed a significant drop in the proportions of memory PbTILs expressing a Th1 phenotype in inguinal lymph nodes compared to spleen (Supplementary Figure 8c)."

3)

In the new figure S8d the authors compare CCR7 expression on memory cells between spleen and LN. However, the cutoff for what is considered + vs – in both organs is not placed at the same CCR7 level. Cells with lower CCR7 level are scored as + in the LN than spleen, which will contribute to having a higher % of CCR7+ cells in LN than spleen in the graph to the right.

The peak of the isotype control seems at the same spot in both organs, so the control is fine and the same cutoff for CCR7+ vs – needs to be applied. It seems that the cutoff was placed at the right end of the isotype distribution in both organs, which is misleading because more cells make up the isotype

peak in the spleen than LN – this of course makes the distribution wider and should therefore not be used to define a cutoff.

Given that this data is not necessary to test the hypothesis that Th1 memory cells are restricted from lymph nodes, we have omitted this analysis, which the Reviewer feels might be confounded by the decision of where to place gates for samples across different tissues, with different numbers of cells in each sample.

Again, this data has now been removed (lines 386-388).

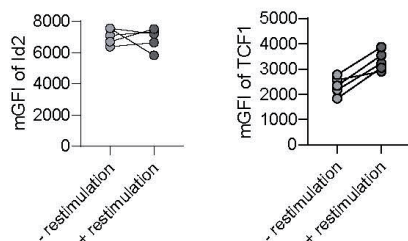
Additional minor points

1) In the new figure S7c it visually seems that the difference between the reference and transfer groups is larger with IAT than saline, nevertheless the * suggest the opposite. Is the visual impression misleading, or is there a chance the * have been accidentally swapped?

We understand the Reviewer's point about statistical testing. We have double checked our statistical analysis and the result holds true. The reason for lower statistical significance for the IAT analysis () versus Saline (**), despite a larger effect, is likely sample size. We had only 4-5 mice per group for IAT, but 6 mice per group for Saline.*

2) According to the methods section, 'To assess cytokine and transcription factor expression, cells were incubated with brefeldin-A (10 mg/ml) with or without ionomycin (500 ng/ml) and PMA...'. Is this a typo? Or were transcription factors indeed assessed after stimulation with PMA/ionomycin? If so, this would be quite unconventional and should therefore be explicitly mentioned in the text, since it would significantly influence the interpretation of the transcription factor data – the stimulation itself may have influenced the expression of the transcription factor and might therefore not at all represent the state of the cells at isolation from the tissue.

We acknowledge the Reviewer's point and propose to explicitly mention the instance where TCF1 was assessed after PMA/ionomycin stimulation (Fig 5g and Supp 8b). All other data relating to transcription factors were performed without stimulation. For the Reviewer's information, we had explicitly examined Id2 and TCF1 in the presence and absence of stimulation with PMA/ionomycin, and present the data below. Stimulation did not alter Id2 staining but did increase in TCF1 expression by PbTII cells at day 28 p.i. (see below).



Samples are PbTII cells from IAT-treated mice at D28 p.i.

This has now been corrected in the manuscript (lines 1026-1032):

“To assess cytokine production and transcription factor expression, cells were incubated with brefeldin-A (10 mg/ml) with or without ionomycin (500 ng/ml) and PMA (25 ng/ml) at 37°C for 3 hours. Intracellular staining for cytokines, transcription factors and chemokines was then performed using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set. Staining with intracellular antibodies was done at 4°C for 1 hour. Where TCF1 was stained after stimulation with PMA and ionomycin for 3 hours, this is explicitly stated figure legends. All other transcription factors and chemokine molecules were stained without prior stimulation.”

In addition, we have now explicitly stated this information in the legends for Figure 5g and Supp 8b where TCF1 was stained after stimulation with PMA/ Ionomycin.

Decision Letter, second revision:**Subject:** Nature Immunology - NI-RS29275B pre-edit**Message:** Our ref: NI-RS29275B

12th Aug 2020

Dear Dr. Haque,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Transcriptome Dynamics of CD4⁺ T cells during Malaria Maps Progressive Transition from Effector to Memory." (NI-RS29275B). Please follow the instructions provided here and in the attached files, as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

When you upload your final materials, please include a point-by-point response to the points below. We won't be able to proceed further without this detailed response.

General formatting:

1. Our standard word limit is 4500 words for the Introduction, Results and Discussion. Your current manuscript greatly exceeds this (nearly double!). We can go to 5000 words at an absolute push.
2. Please include a separate "Data availability" subsection at the end of your Online Methods. This section should inform our readers about the availability of the data used to support the conclusions of your study and should include references to source data, accession codes to public repositories, URLs to data repository entries, dataset DOIs, and any other statement about data availability. We strongly encourage submission of source data (see below) for all your figures. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, these should be included in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: <http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf>.
3. The title should provide a clear and compelling summary of the main findings in fewer than 100 characters including spaces and without punctuation.
4. Your abstract must be fewer than 150 words and should not include citations.
5. As a guideline, Articles allow up to 50 references in the main text. An additional 20 references can be included in the Online Methods. Only papers that have been published or accepted by a named publication or recognized preprint server should be in the numbered list. Published conference abstracts, numbered patents and research data sets that have been assigned a digital object identifier may be included in the reference list.
6. Unpublished meeting abstracts, personal communications and manuscripts under

consideration (and not formally accepted) may be cited only internally within the text and should not be added to the reference list. Please provide names of all authors of unpublished data. If you cite personal communications or unpublished data of any individuals who are not authors of your manuscript, you must supply copies of written permission from the primary investigator of each group cited. Permission in the form of an email will suit this purpose.

7. All references must be cited in numerical order. Place Methods-only references after the Methods section and continue the numbering of the main reference list (i.e., do not start at 1).

8. Equations and symbols that will be set apart from the text must be in an editable format. Do not use embedded images for equations or symbols.

9. Genes must be clearly distinguished from gene products (e.g., "gene *Abc* encodes a kinase," not "gene *Abc* is a kinase"). For genes, provide database-approved official symbols (e.g., NCBI Gene, <http://www.ncbi.nlm.nih.gov/gene>) for the relevant species the first time each is mentioned; gene aliases may be used thereafter. Italicize gene symbols and functionally defined locus symbols; do not use italics for proteins, noncoding gene products and spelled-out gene names.

Figures and Tables:

10. All figures and tables, including Extended Data, must be cited in the text in numerical order.

11. Figure legends should be concise. Begin with a brief title and then describe what is presented in the figure and detail all relevant statistical information, avoiding inappropriate methodological detail.

12. All relevant figures must have scale bars (rather than numerical descriptions of magnification).

13. All relevant figures must have defined error bars.

14. Graph axes should start at zero and not be altered in scale to exaggerate effects. A 'broken' graph can be used if absolutely necessary due to sizing constraints, but the break must be visually evident and should not impinge on any data points.

15. All bar graphs should be converted to a dot-plot format or to a box-and-whisker format to show data distribution. All box-plot elements (center line, limits, whiskers, points) should be defined.

16. When submitting the revised version of your manuscript, please pay close attention to our [Digital Image Integrity Guidelines](https://www.nature.com/nature-research/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel

lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Statistics and Reproducibility:

17. The Methods must include a statistics section where you describe the statistical tests used. For all statistics (including error bars), provide the EXACT n values used to calculate the statistics (reporting individual values rather than a range if n varied among experiments) AND define type of replicates (e.g., cell cultures, technical replicates). Please avoid use of the ambiguous term “biological replicates”; instead state what constituted the replicates (e.g., cell cultures, independent experiments, etc.). For all representative results, indicate number of times experiments were repeated, number of images collected, etc. Indicate statistical tests used, whether the test was one- or two-tailed, exact values for both significant and non-significant P values where relevant, F values and degrees of freedom for all ANOVAs and t-values and degrees of freedom for t-tests.

18. **Reporting Guidelines**– Attached you will find an annotated version of the Reporting Summary you submitted, along with a Word document indicating revisions that need to be made in compliance with our reproducibility requirements. These documents detail any changes that will need to be made to the text, and particularly the main and supplementary figure legends, including (but not limited to) details regarding sample sizes, replication, scale and error bars, and statistics. Please use these documents as a guide when preparing your revision and submit an updated Reporting Summary with your revised manuscript. The Reporting Summary will be published as supplementary material when your manuscript is published.

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25 **EXTENDED DATA:** Extended Data are an integral part of the paper and only data that directly contribute to the main message should be presented. These figures will be integrated into the full-text HTML version of your paper and will be appended to the online

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26 SUPPLEMENTARY INFORMATION: Supplementary Information is material that is essential background to the study but which is not practical to include in the printed version of the paper (for example, video files, large data sets and calculations). Each item must be referred to in the main manuscript and detailed in the attached Inventory of Accessory Information. Tables containing large data sets should be in Excel format, with the table number and title included within the body of the table. All textual information and any additional Supplementary Figures (which should be presented with the legends directly below each figure) should be provided as a single, combined PDF. Please note that we cannot accept resupplies of Supplementary Information after the paper has been formally accepted unless there has been a critical scientific error.

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Other

28 As mentioned in our previous letter, all corresponding authors on a manuscript should have an ORCID – please visit your account in our manuscript system to link your ORCID to your profile, or to create one if necessary. For more information please see our previous letter or visit www.springernature.com/orcid.

29 Nature Research journals [encourage authors to share their step-by-step experimental protocols](https://www.nature.com/nature-research/editorial-policies/reporting-standards#protocols) on a protocol sharing platform of their choice. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can be found at www.nature.com/protocolexchange/about.

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Best regards,

Zoltan Fehervari, Ph.D.

Senior Editor
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Sincerely,

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